BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTI Inte

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 27/00, 27/26

A1

(11) International Publication Number: WO 96/04547

(43) International Publication Date: 15 February 1996 (15.02.96)

(21) International Application Number: PCT/US95/09492

(22) International Filing Date: 1 August 1995 (01.08.95)

(30) Priority Data:
08/283,769 1 August 1994 (01.08.94) US

(71) Applicant (*for all designated States except US*): LOCKHEED MARTIN ENERGY SYSTEMS, INC. [US/US]; P.O. Box 2009, Oak Ridge, TN 37831-8243 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): RAMSEY, J., Michael [US/US]; 733 Hampton Roads Drive, Knoxville, TN 37922 (US).

(74) Agent: SMIRMAN, Preston, H.; Lockheed Martin Energy Systems, Inc., P.O. Box 2009, Oak Ridge, TN 37831-8243 (US).

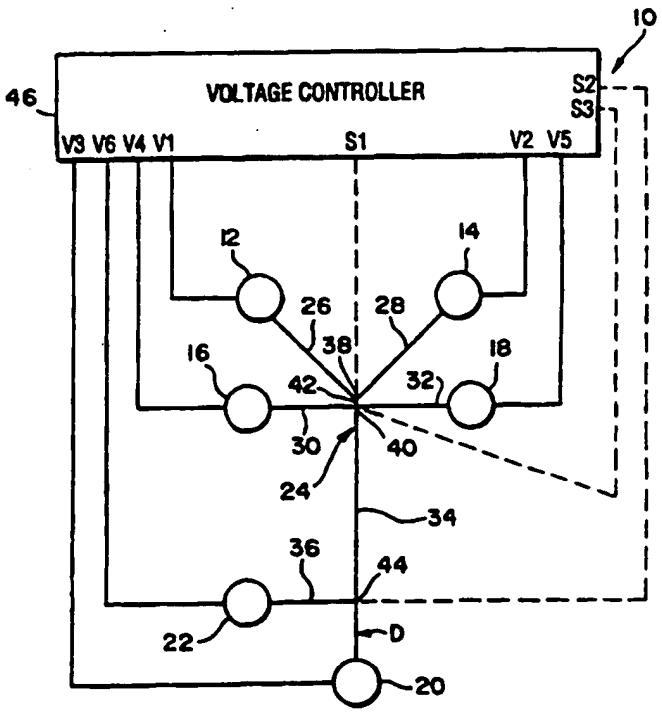
(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: APPARATUS AND METHOD FOR PERFORMING MICROFLUIDIC MANIPULATIONS FOR CHEMICAL ANALYSIS AND SYNTHESIS

(57) Abstract

A microchip loboratory system (10) and method provide fluidic manipulations for a variety of applications, including sample injection for microchip chemical separations. The microchip is fabricated using standard photolithographic procedures and chemical wet etching, with the substrate and cover plate joined using direct bonding. Capillary electrophoresis and electrochromatography are performed in channels (26, 28, 30, 32, 34, 36, 38) formed in the substrate. Analytes are loaded into a four-way intersection of channels by electrokinetically pumping the analyte through the intersection (40), followed by a switching of the potentials to force an analyte plug into the separation channel (34).



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Description

APPARATUS AND METHOD FOR PERFORMING MICROFLUIDIC MANIPULATIONS FOR CHEMICAL ANALYSIS AND SYNTHESIS

This invention was made with Government support under contract DE-AC05-84OR21400 awarded by the U.S. Department of Energy to Martin Marietta Energy Systems, Inc. and the Government has certain rights in this invention.

Field of the invention

The present invention relates generally to miniature instrumentation for chemical analysis, chemical sensing and synthesis and, more specifically, to electrically controlled manipulations of fluids in micromachined channels. These manipulations can be used in a variety of applications, including the electrically controlled manipulation of fluid for capillary electrophoresis, liquid chromatography, flow injection analysis, and chemical reaction and synthesis.

Background of the invention

Laboratory analysis is a cumbersome process. Acquisition of chemical and biochemical information requires expensive equipment, specialized labs and highly trained personnel. For this reason, laboratory testing is done in only a fraction of circumstances where acquisition of chemical information would be useful. A large proportion of testing in both research and clinical situations is done with crude manual methods that are characterized by high labor costs, high reagent consumption, long turnaround times, relative imprecision and poor reproducibility. The practice of techniques such as electrophoresis that are in widespread use in biology and medical laboratories have not changed significantly in thirty years.

Operations that are performed in typical laboratory processes include specimen preparation, chemical/biochemical conversions, sample fractionation, signal detection and data processing. To accomplish these tasks, liquids are often measured and dispensed with volumetric accuracy, mixed together, and subjected to one or several different physical or chemical environments that accomplish conversion or fractionation. In research, diagnostic, or development situations, these operations are carried out on a macroscopic scale using fluid volumes in the range of a few microliters to several liters at a time. Individual operations are performed in series, often using different specialized equipment and instruments for separate steps in the process. Complications, difficulty

and expense are often the result of operations involving multiple laboratory processing steps.

Many workers have attempted to solve these problems by creating integrated laboratory systems. Conventional robotic devices have been adapted to perform pipetting, specimen handling, solution mixing, as well as some fractionation and detection operations. However, these devices are highly complicated, very expensive and their operation requires so much training that their use has been restricted to a relatively small number of research and development programs. More successful have been automated clinical diagnostic systems for rapidly and inexpensively performing a small number of applications such as clinical chemistry tests for blood levels of glucose, electrolytes and gases. Unfortunately due to their complexity, large size and great cost, such equipment, is limited in its application to a small number of diagnostic circumstances.

The desirability of exploiting the advantages of integrated systems in a broader context of laboratory applications has led to proposals that such systems be miniaturized. In the 1980's, considerable research and development effort was put into an exploration of the concept of biosensors with the hope they might fill the need. Such devices make use of selective chemical systems or biomolecules that are coupled to new methods of detection such as electrochemistry and optics to transduce chemical signals to electrical ones that can be interpreted by computers and other signal processing units. Unfortunately, biosensors have been a commercial disappointment. Fewer than 20 commercialized products were available in 1993, accounting for revenues in the U.S. of less than $100 million. Most observers agree that this failure is primarily technological rather than reflecting a misinterpretation of market potential. In fact, many situations such as massive screening for new drugs, highly parallel genetic research and testing, micro-chemistry to minimize costly reagent consumption and waste generation, and bedside or doctor's office diagnostics would greatly benefit from miniature integrated laboratory systems.

In the early 1990's, people began to discuss the possibility of creating miniature versions of conventional technology. Andreas Manz was one of the first to articulate the idea in the scientific press. Calling them "miniaturized total analysis systems," or "μ-TAS," he predicted that it would be possible to integrate into single units microscopic versions of the various elements necessary to process chemical or biochemical samples, thereby achieving automated experimentation. Since that time, miniature components have appeared, particularly molecular separation methods and microvalves. However, attempts to combine these systems into completely integrated

systems have not met with success. This is primarily because precise manipulation of tiny fluid volumes in extremely narrow channels has proven to be a difficult technological hurdle.

One prominent field susceptible to miniaturization is capillary electrophoresis. Capillary electrophoresis has become a popular technique for separating charged molecular species in solution. The technique is performed in small capillary tubes to reduce band broadening effects due to thermal convection and hence improve resolving power. The small tubes imply that minute volumes of materials, on the order of nanoliters, must be handled to inject the sample into the separation capillary tube.

Current techniques for injection include electromigration and siphoning of sample from a container into a continuous separation tube. Both of these techniques suffer from relatively poor reproducibility, and electromigration additionally suffers from electrophoretic mobility-based bias. For both sampling techniques the input end of the analysis capillary tube must be transferred from a buffer reservoir to a reservoir holding the sample. Thus, a mechanical manipulation is involved. For the siphoning injection, the sample reservoir is raised above the buffer reservoir holding the exit end of the capillary for a fixed length of time.

An electromigration injection is effected by applying an appropriately polarized electrical potential across the capillary tube for a given duration while the entrance end of the capillary is in the sample reservoir. This can lead to sampling bias because a disproportionately larger quantity of the species with higher electrophoretic mobilities migrate into the tube. The capillary is removed from the sample reservoir and replaced into the entrance buffer reservoir after the injection duration for both techniques.

A continuing need exists for methods and apparatuses which lead to improved electrophoretic resolution and improved injection stability.

Summary of the Invention

The present invention provides microchip laboratory systems and methods that allow complex biochemical and chemical procedures to be conducted on a microchip under electronic control. The microchip laboratory systems comprises a material handling apparatus that transports materials through a system of interconnected, integrated channels on a microchip. The movement of the materials is precisely directed by controlling the electric fields produced in the integrated channels. The precise control of the movement of such materials enables precise mixing, separation, and reaction as needed to implement a desired biochemical or chemical procedure.

The microchip laboratory system of the present invention analyzes and/or synthesizes chemical materials in a precise and reproducible manner. The system includes a body having integrated channels connecting a plurality of reservoirs that store the chemical materials used in the chemical analysis or synthesis performed by the system. In one aspect, at least five of the reservoirs simultaneously have a controlled electrical potential, such that material from at least one of the reservoirs is transported through the channels toward at least one of the other reservoirs. The transportation of the material through the channels provides exposure to one or more selected chemical or physical environments, thereby resulting in the synthesis or analysis of the chemical material.

The microchip laboratory system preferably also includes one or more intersections of integrated channels connecting three or more of the reservoirs. The laboratory system controls the electric fields produced in the channels in a manner that controls which materials in the reservoirs are transported through the intersection(s). In one embodiment, the microchip laboratory system acts as a mixer or diluter that combines materials in the intersection(s) by producing an electrical potential in the intersection that is less than the electrical potential at each of the two reservoirs from which the materials to be mixed originate. Alternatively, the laboratory system can act as a dispenser that electrokinetically injects precise, controlled amounts of material through the intersection(s).

By simultaneously applying an electrical potential at each of at least five reservoirs, the microchip laboratory system can act as a complete system for performing an entire chemical analysis or synthesis. The five or more reservoirs can be configured in a manner that enables the electrokinetic separation of a sample to be analyzed ("the analyte") which is then mixed with a reagent from a reagent reservoir. Alternatively, a chemical reaction of an analyte and a solvent can be performed first, and then the material resulting from the reaction can be electrokinetically separated. As such, the use of five or more reservoirs provides an integrated laboratory system that can perform virtually any chemical analysis or synthesis.

In yet another aspect of the invention, the microchip laboratory system includes a double intersection formed by channels interconnecting at least six reservoirs. The first intersection can be used to inject a precisely sized analyte plug into a separation channel toward a waste reservoir. The electrical potential at the second intersection can be selected in a manner that provides additional control over the size of the analyte plug. In addition, the electrical potentials can be controlled in a manner that transports materials from the fifth and sixth reservoirs through the second intersection toward the

first intersection and toward the fourth reservoir after a selected volume of material from the first intersection is transported through the second intersection toward the fourth reservoir. Such control can be used to push the analyte plug further down the separation channel while enabling a second analyte plug to be injected through the first intersection.

In another aspect, the microchip laboratory system acts as a microchip flow control system to control the flow of material through an intersection formed by integrated channels connecting at least four reservoirs. The microchip flow control system simultaneously applies a controlled electrical potential to at least three of the reservoirs such that the volume of material transported from the first reservoir to a second reservoir through the intersection is selectively controlled solely by the movement of a material from a third reservoir through the intersection. Preferably, the material moved through the third reservoir to selectively control the material transported from the first reservoir is directed toward the same second reservoir as the material from the first reservoir. As such, the microchip flow control system acts as a valve or a gate that selectively controls the volume of material transported through the intersection. The microchip flow control system can also be configured to act as a dispenser that prevents the first material from moving through the intersection toward the second reservoir after a selected volume of the first material has passed through the intersection. Alternatively, the microchip flow control system can be configured to act as a diluter that mixes the first and second materials in the intersection in a manner that simultaneously transports the first and second materials from the intersection toward the second reservoir.

Other objects, advantages and salient features of the invention will become apparent from the following detailed description, which taken in conjunction with the annexed drawings, discloses preferred embodiments of the invention.

Brief Description of the Drawings

Figure 1 is a schematic view of a preferred embodiment of the present invention;

Figure 2 is an enlarged, vertical sectional view of a channel shown;

Figure 3 is a schematic, top view of a microchip according to a second preferred embodiment of the present invention;

Figure 4 is an enlarged view of the intersection region of Figure 3;

Figure 5 are CCD images of a plug of analyte moving through the intersection of the Figure 30 embodiment;

Figure 6 is a schematic top view of a microchip laboratory system according to a third preferred embodiment of a microchip according to the present invention;

Figure 7 is a CCD image of "sample loading mode for rhodamine B" (shaded area);

Figure 8(a) is a schematic view of the intersection area of the microchip of Figure 6, prior to analyte injection;

Figure 8(b) is a CCD fluorescence image taken of the same area depicted in Figure 8(a), after sample loading in the pinched mode;

Figure 8(c) is a photomicrograph taken of the same area depicted in Figure 8(a), after sample loading in the floating mode;

Figure 9 shows integrated fluorescence signals for injected volume plotted versus time for pinched and floating injections;

Figure 10 is a schematic, top view of a microchip according to a fourth preferred embodiment of the present invention;

Figure 11 is an enlarged view of the intersection region of Figure 10;

Figure 12 is a schematic top view of a microchip laboratory system according to a fifth preferred embodiment according to the present invention;

Figure 13(a) is a schematic view of a CCD camera view of the intersection area of the microchip laboratory system of Figure 12;

Figure 13(b) is a CCD fluorescence image taken of the same area depicted in Figure 13(a), after sample loading in the pinched mode;

Figures 13(c)-13(e) are CCD fluorescence images taken of the same area depicted in Figure 13(a), sequentially showing a plug of analyte moving away from the channel intersection at 1, 2, and 3 seconds, respectively, after switching to the run mode;

Figure 14 shows two injection profiles for didansyl-lysine injected for 2s with γ equal to 0.97 and 9.7;

Figure 15 are electropherograms taken at (a) 3.3 cm, (b) 9.9 cm, and (c) 16.5 cm from the point of injection for rhodamine B (less retained) and sulforhodamine (more retained);

Figure 16 is a plot of the efficiency data generated from the electropherograms of Figure 15, showing variation of the plate number with.. channel length for rhodamine B (square with plus) and sulforhodamine (square with plus) and sulforhodamine (square with dot) with best linear fit (solid lines) for each analyte;

Figure 17(a) is an electropherogram of rhodamine B and fluorescein with a separation field strength of 1.5 kV/cm and a separation length of 0.9 mm;

Figure 17(b) is an electropherogram of rhodamine B and fluorescein with a separation field strength of 1.5 kV/cm and a separation length of 1.6 mm;

Figure 17(c) is an electropherogram of rhodamine B and fluorescein with a separation field strength of 1.5 kV/cm and a separation length of 11.1 mm;

Figure 18 is a graph showing variation of the number of plates per unit time as a function of the electric field strength for rhodamine B at separation lengths of 1.6 mm (circle) and 11.1 mm (square) and for fluorescein at separation lengths of 1.6 mm (diamond) and 11.1 mm (triangle);

Figure 19 shows a chromatogram of coumarins analyzed by electrochromatography using the system of Figure 12;

Figure 20 shows a chromatogram of coumarins resulting from micellar electrokinetic capillary chromatography using the system of Figure 12;

Figures 21(a) and 21(b) show the separation of three metal ions using the system of Figure 12;

Figure 22 is a schematic, top plan view of a microchip according to the Figure 3 embodiment, additionally including a reagent reservoir and reaction channel;

Figure 23 is a schematic view of the embodiment of Figure 20, showing applied voltages;

Figure 24 shows two electropherograms produced using the Figure 22 embodiment;

Figure 25 is a schematic view of a microchip laboratory system according to a sixth preferred embodiment of the present invention;

Figure 26 shows the reproducibility of the amount injected for arginine and glycine using the system of Figure 25;

Figure 27 shows the overlay of three electrophoretic separations using the system of Figure 25;

Figure 28 shows a plot of amounts injected versus reaction time using the system of Figure 25;

Figure 29 shows an electropherogram of restriction fragments produced using the system of Figure 25;

Figure 30 is a schematic view of a microchip laboratory system according to a seventh preferred embodiment of the present invention.

Figure 31 is a schematic view of the apparatus of Figure 21, showing sequential applications of voltages to effect desired fluidic manipulations; and

Figure 32 is a graph showing the different voltages applied to effect the fluidic manipulations of Figure 23.

Detailed Description of the Invention

Integrated, micro-laboratory systems for analyzing or synthesizing chemicals require a precise way of manipulating fluids and fluid-borne material and subjecting the fluids to selected chemical or physical environments that produce desired conversions or partitioning. Given the concentration of analytes that produces chemical conversion in reasonable time scales, the nature of molecular detection, diffusion times and manufacturing methods for creating devices on a microscopic scale, miniature integrated micro-laboratory systems lend themselves to channels having dimensions on the order of 1 to 100 micrometers in diameter. Within this context, electrokinetic pumping has proven to be versatile and effective in transporting materials in microfabricated laboratory systems.

The present invention provides the tools necessary to make use of electrokinetic pumping not only in separations, but also to perform liquid handling that accomplishes other important sample processing steps, such as chemical conversions or sample partitioning. By simultaneously controlling voltage at a plurality of ports connected by channels in a microchip structure, it is possible to measure and dispense fluids with great precision, mix reagents, incubate reaction components, direct the components towards sites of physical or biochemical partition, and subject the components to detector systems. By combining these capabilities on a single microchip, one is able to create complete, miniature, integrated automated laboratory systems for analyzing or synthesizing chemicals.

Such integrated micro-laboratory systems can be made up of several component elements. Component elements can include liquid dispersing systems, liquid mixing systems, molecular partition systems, detector sights, etc. For example, as described herein, one can construct a relatively complete system for the identification of restriction endonuclease sites in a DNA molecule. This single microfabricated device thus includes in a single system the functions that are traditionally performed by a technician employing pipettors, incubators, gel electrophoresis systems, and data acquisition systems. In this system, DNA is mixed with an enzyme, the mixture is incubated, and a selected volume of the reaction mixture is dispensed into a separation channel. Electrophoresis is conducted concurrent with fluorescent labeling of the DNA.

Shown in Figure 1 is an example of a microchip laboratory system 10 configured to implement an entire chemical analysis or synthesis. The laboratory system 10 includes six reservoirs 12, 14, 16, 18, 20, and 22 connected to each other by a system of channels 24 micromachined into a substrate or base member (not shown in Fig. 1), as

discussed in more detail below. Each reservoir 12-22 is in fluid communication with a corresponding channel 26, 28, 30, 32, 34, 36, and 38 of the channel system 24. The first channel 26 leading from the first reservoir 12 is connected to the second channel 28 leading from the second reservoir 14 at a first intersection 38. Likewise, the third channel 30 from the third reservoir 16 is connected to the fourth channel 32 at a second intersection 40. The first intersection 38 is connected to the second intersection 40 by a reaction chamber or channel 42. The fifth channel 34 from the fifth reservoir 20 is also connected to the second intersection 40 such that the second intersection 40 is a four-way intersection of channels 30, 32, 34, and 42. The fifth channel 34 also intersects the sixth channel 36 from the sixth reservoir 22 at a third intersection 44

The materials stored in the reservoirs preferably are transported electrokinetically through the channel system 24 in order to implement the desired analysis or synthesis. To provide such electrokinetic transport, the laboratory system 10 includes a voltage controller 46 capable of applying selectable voltage levels, including ground. Such a voltage controller can be implemented using multiple voltage dividers and multiple relays to obtain the selectable voltage levels. The voltage controller is connected to an electrode positioned in each of the six reservoirs 12-22 by voltage lines V1-V6 in order to apply the desired voltages to the materials in the reservoirs. Preferably, the voltage controller also includes sensor channels S1, S2, and S3 connected to the first, second, and third intersections 38, 40, 44, respectively, in order to sense the voltages present at those intersections.

The use of electrokinetic transport on microminiaturized planar liquid phase separation devices, described above, is a viable approach for sample manipulation and as a pumping mechanism for liquid chromatography. The present invention also entails the use of electroosmotic flow to mix various fluids in a controlled and reproducible fashion. When an appropriate fluid is placed in a tube made of a correspondingly appropriate material, functional groups at the surface of the tube can ionize. In the case of tubing materials that are terminated in hydroxyl groups, protons will leave the surface and enter an aqueous solvent. Under such conditions the surface will have a net negative charge and the solvent will have an excess of positive charges, mostly in the charged double layer at the surface. With the application of an electric field across the tube, the excess cations in solution will be attracted to the cathode, or negative electrode. The movement of these positive charges through the tube will drag the solvent with them. The steady state velocity is given by equation 1,

$$v = \frac{\varepsilon \xi E}{4 \pi \eta} \qquad (1)$$

where v is the solvent velocity, ε is the dielectric constant of the fluid, ξ is the zeta potential of the surface, E is the electric field strength, and π is the solvent viscosity. From equation 1 it is obvious that the fluid flow velocity or flow rate can be controlled through the electric field strength. Thus, electroosmosis can be used as a programmable pumping mechanism.

The laboratory microchip system 10 shown in Figure 1 could be used for performing numerous types of laboratory analysis or synthesis, such as DNA sequencing or analysis, electrochromatography, micellar electrokinetic capillary chromatography (MECC), inorganic ion analysis, and gradient elution liquid chromatography, as discussed in more detail below. The fifth channel 34 typically is used for electrophoretic or electrochromatographic separations and thus may be referred to in certain embodiments as a separation channel or column. The reaction chamber 42 can be used to mix any two chemicals stored in the first and second reservoirs 12, 14. For example, DNA from the first reservoir 12 could be mixed with an enzyme from the second reservoir 14 in the first intersection 38 and the mixture could be incubated in the reaction chamber 42. The incubated mixture could then be transported through the second intersection 40 into the separation column 34 for separation. The sixth reservoir 22 can be used to store a fluorescent label that is mixed in the third intersection 44 with the materials separated in the separation column 34. An appropriate detector (D) could then be employed to analyze the labeled materials between the third intersection 44 and the fifth reservoir 20. By providing for a pre-separation column reaction in the first intersection 38 and reaction chamber 42 and a post-separation column reaction in the third intersection 44, the laboratory system 10 can be used to implement many standard laboratory techniques normally implemented manually in a conventional laboratory. In addition, the elements of the laboratory system 10 could be used to build a more complex system to solve more complex laboratory procedures.

The laboratory microchip system 10 includes a substrate or base member (not shown in Fig. 1) which can be an approximately two inch by one inch piece of microscope slide (Corning, Inc. #2947). While glass is a preferred material, other similar materials may be used, such as fused silica, crystalline quartz, fused quartz, plastics, and silicon (if the surface is treated sufficiently to alter its resistivity). Preferably, a non-conductive material such as glass or fused quartz is used to allow relatively high electric fields to be applied to electrokinetically transport materials through channels in the microchip. Semiconducting materials such as silicon could also be used, but the electric field applied would normally need to be kept to a minimum (approximately less than 300

volts per centimeter using present techniques of providing insulating layers), which may provide insufficient electrokinetic movement.

The channel pattern 24 is formed in a planar surface of the substrate using standard photolithographic procedures followed by chemical wet etching. The channel pattern may be transferred onto the substrate with a positive photoresist (Shipley 1811) and an e-beam written chrome mask (Institute of Advanced Manufacturing Sciences, Inc.). The pattern may be chemically etched using HF/NH_4F solution

After forming the channel pattern, a cover plate may then be bonded to the substrate using a direct bonding technique whereby the substrate and the cover plate surfaces are first hydrolyzed in a dilute NH_4OH/H_2O_2 solution and then joined. The assembly is then annealed at about 500° C in order to insure proper adhesion of the cover plate to the substrate.

Following bonding of the cover plate, the reservoirs are affixed to the substrate, with portions of the cover plate sandwiched therebetween, using epoxy or other suitable means. The reservoirs can be cylindrical with open opposite axial ends. Typically, electrical contact is made by placing a platinum wire electrode in each reservoirs. The electrodes are connected to a voltage controller 46 which applies a desired potential to select electrodes, in a manner described in more detail below.

A cross section of the first channel is shown in Figure 2 and is identical to the cross section of each of the other integrated channels. When using a non-crystalline material (such as glass) for the substrate, and when the channels are chemically wet etched, an isotropic etch occurs, *i.e.*, the glass etches uniformly in all directions, and the resulting channel geometry is trapezoidal. The trapezoidal cross section is due to "undercutting" by the chemical etching process at the edge of the photoresist. In one embodiment, the channel cross section of the illustrated embodiment has dimensions of 5.2 μm in depth, 57 μm in width at the top and 45 μm in width at the bottom. In another embodiment, the channel has a depth "d" of 10μm, an upper width "w1" of 90μm, and a lower width "w2" of 70μm.

An important aspect of the present invention is the controlled electrokinetic transportation of materials through the channel system 24. Such controlled electrokinetic transport can be used to dispense a selected amount of material from one of the reservoirs through one or more intersections of the channel structure 24. Alternatively, as noted above, selected amounts of materials from two reservoirs can be transported to an intersection where the materials can be mixed in desired concentrations.

Gated Dispenser

Shown in Figure 3 is a laboratory component 10A that can be used to implement a preferred method of transporting materials through a channel structure 24A. The A following each number in Figure 3 indicates that it corresponds to an analogous element of Figure 1 of the same number without the A. For simplicity, the electrodes and the connections to the voltage controller that controls the transport of materials through the channel system 24A are not shown in Figure 3.

The microchip laboratory system 10A shown in Figure 3 controls the amount of material from the first reservoir 12A transported through the intersection 40A toward the fourth reservoir 20A by electrokinetically opening and closing access to the intersection 40A from the first channel 26A. As such, the laboratory microchip system 10A essentially implements a controlled electrokinetic valve. Such an electrokinetic valve can be used as a dispenser to dispense selected volumes of a single material or as a mixer to mix selected volumes of plural materials in the intersection 40A. In general, electro-osmosis is used to transport "fluid materials" and electrophoresis is used to transport ions without transporting the fluid material surrounding the ions. Accordingly, as used herein, the term "material" is used broadly to cover any form of material, including fluids and ions.

The laboratory system 10A provides a continuous unidirectional flow of fluid through the separation channel 34A. This injection or dispensing scheme only requires that the voltage be changed or removed from one (or two) reservoirs and allows the fourth reservoir 20A to remain at ground potential. This will allow injection and separation to be performed with a single polarity power supply.

An enlarged view of the intersection 40A is shown in Figure 4. The directional arrows indicate the time sequence of the flow profiles at the intersection 40A. The solid arrows show the initial flow pattern. Voltages at the various reservoirs are adjusted to obtain the described flow patterns. The initial flow pattern brings a second material from the second reservoir 16A at a sufficient rate such that all of the first material transported from reservoir 12A to the intersection 40A is pushed toward the third reservoir 18A. In general, the potential distribution will be such that the highest potential is in the second reservoir 16A, a slightly lower potential in the first reservoir 12A, and yet a lower potential in the third reservoir 18A, with the fourth reservoir 20A being grounded. Under these conditions, the flow towards the fourth reservoir 20A is solely the second material from the second reservoir 16A.

To dispense material from the first reservoir 12A through the intersection 40A, the potential at the second reservoir 16A can be switched to a value less than the

potential of the first reservoir 12A or the potentials at reservoirs 16A and/or 18A, can be floated momentarily to provide the flow shown by the short dashed arrows in Figure 4. Under these conditions, the primary flow will be from the first reservoir 12A down towards the separation channel waste reservoir 20A. The flow from the second and third reservoirs 16A, 18A will be small and could be in either direction. This condition is held long enough to transport a desired amount of material from the first reservoir 12A through the intersection 40A and into the separation channel 34A. After sufficient time for the desired material to pass through the intersection 40A, the voltage distribution is switched back to the original values to prevent additional material from the first reservoir 12A from flowing through the intersection 40A toward the separation channel 34A.

One application of such a "gated dispenser" is to inject a controlled, variable-sized plug of analyte from the first reservoir 12A for electrophoretic or chromatographic separation in the separation channel 34A. In such a system, the first reservoir 12A stores analyte, the second reservoir 16A stores an ionic buffer, the third reservoir 18A is a first waste reservoir and the fourth reservoir 20A is a second waste reservoir. To inject a small variable plug of analyte from the first reservoir 12A, the potentials at the buffer and first waste reservoirs 16A, 18A are simply floated for a short period of time (≈ 100 ms) to allow the analyte to migrate down the separation column 34A. To break off the injection plug, the potentials at the buffer reservoir 16A and the first waste reservoir 18A are reapplied. Alternatively, the valving sequence could be effected by bringing reservoirs 16A and 18A to the potential of the intersection 40A and then returning them to their original potentials. A shortfall of this method is that the composition of the injected plug has an electrophoretic mobility bias whereby the faster migrating compounds are introduced preferentially into the separation column 34A over slower migrating compounds.

In Figure 5, a sequential view of a plug of analyte moving through the intersection of the Figure 3 embodiment can be seen by CCD images. The analyte being pumped through the laboratory system 10A was rhodamine B (shaded area), and the orientation of the CCD images of the injection cross or intersection is the same as in Figure 3. The first image, (A), shows the analyte being pumped through the injection cross or intersection toward the first waste reservoir 18A prior to the injection. The second image, (B), shows the analyte plug being injected into the separation column 34A. The third image, (C), depicts the analyte plug moving away from the injection intersection after an injection plug has been completely introduced into the separation column 34A. The potentials at the buffer and first waste reservoirs 16A, 18A were

DORSEY & WHITNEY LLP

MINNEAPOLIS
SEATTLE
NEW YORK
WASHINGTON, D.C.
DENVER
LONDON
SAN FRANCISCO
ANCHORAGE
DES MOINES
SALT LAKE CITY
SOUTHERN CALIFORNIA
FARGO

FOUR EMBARCADERO CENTER
SUITE 3400
SAN FRANCISCO, CALIFORNIA 94111-4187
TELEPHONE: (415) 781-1989
FAX: (415) 398-3249
www.dorseylaw.com

BRUSSELS
TOKYO
PALO ALTO
GREAT FALLS
ROCHESTER
TORONTO
HONG KONG
MISSOULA
NORTHERN VIRGINIA
SHANGHAI
VANCOUVER

FAX COVER SHEET

DATE:	**September 5, 2002**	TOTAL # OF PAGES (INCLUDING THIS COVER SHEET):	**61**
TO:	**Omar D. Perez, Ph.D.**	FAX #:	**650-723-2383**
FIRM NAME:	**Baxter Laboratory of Genetic Pharmacology Stanford University School of Medicine 269 Campus Dr., CCSR 3220 Stanford, CA 94305-5175**	TELEPHONE #:	**650-724-4847**
FROM:	**Anna Gil**	FAX #:	**(415) 398-3249**
TELEPHONE #:	**(415) 781-1989**	EMAIL:	**gil.anna@dorseylaw.com**
YOUR REF. NO.:	**SO1-069**		
OUR REF. NO.:	**468330-01773 (A-70545)**		

RE: FIGURES 1-39 AND SUPPLEMENTAL FIGURES
United States Patent Application
Serial No.: 10/193,462
Filing Date: July 10, 2002
Title: Methods and Compositions for Detecting the Activation State of Multiple Proteins in Single Cells
Inventors: Omar D. Perez and Garry P. Nolan

Dear Omar:

Attached are the Figures, and their description, as filed with the above-identified application. Please note that Figures 11 and 12 are missing and should be included, if available. Also, please advise us on whether the Supplementary Figures can be combined with the corresponding non-supplemental Figures.

Thank you for your help regarding this matter.

If you have any questions re this application, please do not hesitate to call me or Robin.

Original will be sent via: ☐ Mail ☐ Messenger ☐ Air Courier ☒ Will not be sent

PLEASE CONTACT Anna Gil AT (415) 781-1989 IF THIS TRANSMISSION IS INCOMPLETE OR CANNOT BE READ.

DORSEY & WHITNEY LLP

MINNEAPOLIS
SEATTLE
NEW YORK
WASHINGTON, D.C.
DENVER
LONDON
SAN FRANCISCO
ANCHORAGE
DES MOINES
SALT LAKE CITY
SOUTHERN CALIFORNIA
FARGO

FOUR EMBARCADERO CENTER
SUITE 3400
SAN FRANCISCO, CALIFORNIA 94111-4187
TELEPHONE: (415) 781-1989
FAX: (415) 398-3249
www.dorseylaw.com

BRUSSELS
TOKYO
PALO ALTO
GREAT FALLS
ROCHESTER
TORONTO
HONG KONG
MISSOULA
NORTHERN VIRGINIA
SHANGHAI
VANCOUVER

FAX COVER SHEET

Regards,

Anna Gil

Original will be sent via: ☐ Mail ☐ Messenger ☐ Air Courier ☒ Will not be sent

PLEASE CONTACT Anna Gil AT (415) 781-1989 IF THIS TRANSMISSION IS INCOMPLETE OR CANNOT BE READ.